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(54) IMPROVED LIMULUS AMEBOCYTE LYSATE REAGENT COMPOSITIONS

(71) We, MALLINCKRODT, INC., a Corporation organised and existing under the laws of the State of Missouri, United States of America, of 675 Brown Road, St. Louis, State of Missouri 63134, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to improved Limulus amebocyte lysate reagent compositions for detecting endotoxin in biological and parenteral fluids and to methods for preparing such compositions.

The hemolymph of the horseshoe crab, *Limulus polyphemus*, contains only one type of cell, viz., amebocytes. Lysis of these amebocytes (or of corresponding cells from certain closely related species) liberates a substance which reacts with endotoxin to form a gel. This reaction between Limulus amebocyte lysate and endotoxin has been made the basis for exquisitely sensitive *in vitro* methods for detecting and/or determining endotoxin in a variety of fluids of pharmaceutical/medical interest. Levin and Bang — "Clottable Protein in Limulus: Its Localization and Kinetics of Its Coagulation by Endotoxin", Thromb. Diath. Haemorrh *19:* 186 — 197 (1968) — described the preparation of Limulus amebocyte lysate. Levin U. S. Patent 3,915,805 (1975) discloses a method of detecting endotoxin in protein-containing material utilizing this lysate.

E. T. Yin, et al. — "Picogram-sensitive Assay for Endotoxin: Gelatin of *Limulus polyphemus* Blood Cell-Lysate Induced by Purified Lipopolysaccharides and Lipid A from Gram-negative Bacteria," Biochim. Biophys. Acta, *261:*284—289 (1972) — carried out lysis of the Limulus amebocytes by mechanical disruption of the cells in a tromethamine (tris) buffer containing calcium and sodium chlorides. They reported that these variations from Levin's procedures yielded a lysate of improved sensitivity and stability.

H. D. Hochstein, et al., — "Further Developments of Limulus Amebocyte Lysate Test," Bull. Par. Drug Assn. *27,* 139—148 (1973) — described several methods of lysing Limulus amebocytes, including the use of ultrasonic waves ("sonification") to disrupt the cells suspended in a tromethamine buffer. They also disclosed that dilution of the lysate with distilled water increased its sensitivity, maximum sensitivity being reached at a dilution of 1 plus 5. They further reported that at room temperature for a month or more the normally white lyophilized lysate discolors and loses activity.

Sullivan and Watson — "Factors Affecting the Sensitivity of *Limulus* Lysate", Applied Microbiology *28:* 1023—1026 (1974) — improved the sensitivity of the lysate toward endotoxin by extracting an inhibitor (using chloroform or other solvents) and by adding low concentrations of divalent cations (Ca^{+2}, Mg^{+2}, Mn^{+2}). They also added sodium chloride to decrease the turbidity in the blank. These investigators also reported that chloroform extraction adversely affected the stability of the lysate. Their reported stability data have been tabulated below in Table 1.

TABLE 1

Lysate Stability as Reported by Sullivan and Watson

Treatment of Lysate	Storage Temp.	Storage Temp.	Effect on Activity
No special treatment	–74°C.	12. mo.	No loss of activity
No special treatment	5°C	> 1 wk.	No loss of activity
Extraction with $CHCl_3$	–74°C.	3—6 mo.	Occasional decrease
Extraction with $CHCl_3$	Room Temp.		Unstable
Extraction with $CHCl_3$	5°C.	Overnight	Frequent loss
Extraction with, $CHCl_3$ and lyophilized			More stable than non-$CHCl_3$ extracted lyophilized lysate
Lyophilized		> 12 mos.	No loss of activity
Lyophilized	37°C.	2 mos.	No loss of activity

The impetus for this investigation came from observations of significant variation in the sensitivity of lysate prepared from horseshoe crabs bled at Woods Hole, Massachusetts, during different years and at different times within a given year. Similarly, M. Kobayashi and M. Yamamoto — "Studies on The Gelation Reaction of Limulus Lysate (Pregel) IV: Effect of Alkaline Earth Metals and Chelating Agents on the Gelation Reaction of Limulus Lysate", Yakugaku Zasshi *95*, 959—965 (1975) — added low concentrations of alkaline earth ions (Mg^{+2}, Ca^{+2}, Ba^{+2}, or Sr^{+2}) to improve the sensitivity of the lysate/endotoxin reaction. R. E. Hopkins (DOS 2,530,685/1976) increased the sensitivity of the lysate to endotoxin by adding to the lysate solution one or more of the following as catalysts; manganese, strontium or barium ions, imidazole, a dithiol (Cleland's Reagent), a monothiol (cysteine) or lithium ions. B. A. Buhler (DOS 2,517,785/1975) approached the same objective by passing the lysate through a 2 micron filter.

J. Eibert — "Pyrogen Testing: Horseshoe Crabs vs. Rabbits", Bull, Par. Drug Assn., *26*, 253—260 (1972) — reported excellent correlation between the Limulus lysate test and the conventional rabbit pyrogen test on a variety of commercial parenteral preparations, including 10% weight/volume fructose and 25% weight/volume mannitol solutions. He also mentioned that fructose solution often contains pyrogens, reflecting the difficulty of removing the last detectable traces of pyrogen from this product.

M. Kobayashi et al — "Studies on the Gelation Reaction of Limulus Lysate (Pre-Gel) III: Influences of Various Substances on the Gelation Reaction of Limulus Lysate", Yakugaku Zasshi *95*: 720—727 (1975) — reported the effects of a number of substances, including glucose and maltose, on the endotoxin-lysate gelation reaction. They found no variations in sensitivity in maltose solutions of 2.7 to 43.2% weight/volume concentration and in glucose solutions of 2.5 to 25% weight/volume concentration. However, the sensitivity was lower in 50% weight/volume glucose solution. Further, they detected endotoxin at various stages in the manufacture of certain products containing maltose.

J. D. Sullivan, et al — "Purification and Properties of the Clotting Enzyme from Limulus Lysate", Biochemical and Biophysical Research Communications, *66:* 848—855 (1975) — isolated from lysate a heat labile enzyme which mediates the endotoxin-clottable protein gelation reaction. Other investigators have described further variations in methods of using Limulus amebocyte lysate and in its applications. See, for example J.F. Cooper, et al — "The Limulus Test for Endotoxin (pyrogen) in Radiopharmaceuticals and Biologics"; Bull. Parenteral Drug Assn. *26:* 153—162 (1972) — and Harris et al U.S. Patent 3,944,391 (1976).

All of the above publications and the references cited therein are incorporated herein by reference.

The solution obtained by lysis of the amebocytes from Limulus hemolymph is not very stable, i.e., its ability to gel in the presence of endotoxin deteriorates rather rapidly at ambient temperatures. This is consistent with the finding referred to above that the lysate contains a heat labile enzyme which is an essential factor in the clotting mechanism. On the other hand, the stability of lysate is greatly enhanced at reduced temperatures. Levin (U. S. 3,915,805) reported that lysate may be stored for a number of months at 4° C. without significant loss of potency.

Limulus amebocyte lysate in lypholized form has been commercially available for some time. The lyophilized lysate is more stable than the liquid lysate and is thus better suited to the conditions necessarily incident to commercial distribution and use. Typically, 1.2 ml. of lysate is lyophilized in a serum vial. The sealed vial with its dry residue is ordinarily stored at low temperatures (below 5° C.) to minimize deterioration of the lysate. In use, such a lyophilized lysate may be reconstituted with 1.2 ml. of sterile, pyrogen-free water. Dissolution of the dry lysate residue is not instantaneous but it is usually accomplished through gentle swirling of the vial and its contents for about 30 seconds.

The reconstituted lysate is aseptically dispensed in 0.1 ml. portions into small test tubes and mixed with 0.1 ml. portions of the solution being tested for endotoxin. The formation of a gel indicates the presence of endotoxin.

The sensitivity of a lysate preparation toward endotoxin may be evaluated by means of its reaction with serially diluted standard endotoxin preparations. A preparation of *E. coli* endotoxin has been found very useful for this purpose. Another widely used endotoxin standard, adopted by the U.S. Food and Drug Administration (FDA), is an endotoxin derived from *Klebsiella pneumoniae*. While Limulus lysate is much more sensitive to the *E. coli* endotoxin than to the FDA standard, both endotoxins are widely used in evaluation lysate preparations.

The present invention provides a reagent composition for detecting endotoxin comprising a lyophilized mixture of Limulus amebocyte lysate and an inert water-soluble, organic stabilizing agent. In one of its aspects the invention is directed to such compositions which additionally include a source of divalent cations. Preferred compositions contain the lysate, a source of divalent cations and a salt of an alkali metal, as well as the stabilizing agent.

The invention also includes a method of preparing the improved reagent compositions of the invention which comprises preparing a Limulus amebocyte lysate solution containing a small proportion of an inert water-soluble, organic stabilizing agent and lyophilizing that solution.

The lyophilized Limulus amebocyte lysate compositions of the present invention generally possess improved thermal stability and high sensitivity.

The inert, water-soluble, organic stabilizing agent used in the compositions of the invention is preferably a crystallizable sugar or a polyhydric alcohol.

The sugars that may be used include the monosaccharides, such as the pentoses, e.g., arabinose, lyxose, ribose, xylose, and rhamnose, and the crystallizable hexoses, e.g., glucose, mannose, galactose, fructose and sorbose. Either the D- or L- forms of the racemic mixtures may be used. Useful sugars also include the disaccharides, e.g., maltose, cellobiose, gentiobiose, lactose, melibiose, primverose, vicianose, trehalose and sucrose. Also useful are the trisaccharides, e.g., melezitose, gentianose and raffinose. Water-soluble, crystallizable polysaccharides, such as inulin, may also be used.

Illustrative of the polyhydric alcohols that may be used are ribitol, xylitol, arabitol, dulcitol, mannitol, iditol, sorbitol, talitol, and inositol.

The disaccharides, especially lactose, are preferred stabilizing agents.

Other inert, water-soluble, organic stabilizing agents useful in the compositions of the invention will occur to those skilled in the art.

As a general rule, the proportion of stabilizing agent used is not critical. Only a small proportion is needed. Ordinarily, a proportion within the range of 0.01%—0.1% weight/volume of stabilizer in the lysate solution prior to lyophilization will achieve the desired end. Lyophilization is facilitated by the use of a non-hygroscopic stabilizer.

Sensitivity of the compositions toward endotoxin is increased by including low concentrations of divalent and monovalent cations. Calcium ions are the preferred divalent ions, although other alkaline earth ions such as magnesium ions or other divalent ions may be used. Sodium ions are the preferred monovalent ions, but other monovalent ions, especially alkali metal ions such as lithium ions may be

used. The chlorides ($CaCl_2$, NaCl, etc.) are convenient sources of these added ions, although other salts may be used. Preferably these electrolytes are added in amounts such that when the lyophilized lysate is reconstituted the divalent cation (e.g., Ca^{+2}) concentration will be in the range of 0.0001 — 0.1 molar and the monovalent cation (e.g., Na^+) concentration will be in the range of 0.001 — 0.1 molar.

All of the additives must, of course, be free of endotoxin. Methods of insuring freedom from endotoxins are known to the art. For example, inorganic additives ($CaCl_2$, NaCl) may be rendered endotoxin-free by heating the dry salts at 250° C. for at least 120 minutes. Organic additives, because of their melting points, etc. must ordinarily be dissolved, and the solution autoclaved at 250°F. for 60 minutes or more to destroy any endotoxins present.

The following example further illustrates the invention.

EXAMPLE

Note: all of the operations described are carried out under conditions such as to insure that the final product is sterile and free of endotoxin.

The hemolymph from healthy specimens of *Limulus polyphemus* is collected in a saline anticoagulant solution generally as described by Levin and Bang (1969 — see above). The amebocytes are collected and washed with the saline anticoagulant solution in a centrifuge.

The separated amebocytes are suspended in water and the osmotic disruption of the cells is complemented by multiple exposures to a combination of mechanical and ultrasonic agitation. The cellular debris is separated from the lysate by means of a centrifuge and the lysate fractions are pooled and stored at 0—4° C.

The sensitivity of the lysate toward endotoxin is determined by the method described later, and if necessary, is adjusted to the desired level of sensitivity by dilution or by mixing with another batch of lysate of different sensitivity. The solution is buffered to the pH range 6.5 — 7.5 by means of tromethamine [tris-(hydroxymethyl) aminomethane] and tromethamine hydrochloride and is made 0.01 molar in calcium chloride, 0.077 molar in sodium chloride and 0.0015 molar (0.05% w/v) in lactose, all of the additives having previously been rendered free of endotoxins.

The buffered lysate solution, prepared as described above, is subdivided into serum vials, each containing 1.2 or 5.2 ml. of the solution, and the subdivided solution is lyophilized. After lyophilization the vials are sealed and refrigerated (1—5° C.).

The lyophilized lysate has the form of a white powder or a white, frangible pellet.

Demonstration of Improved Stability

A lot of lyophilized lysate was prepared as described in the above example, from horseshoe crabs caught near Wallops Island, Virginia. It was designated "L". Two other lots, prepared similarly except that the lactose was omitted, were designated "U" and "A", respectively.

Storage tests at two different elevated temperatures were conducted on tubes from each of the three lots. Periodically, the lyophilized residues in tubes selected at random from each lot were redissolved and the sensitivity of the reconstituted lysate was evaluated by means of the gelation reaction with standard endotoxin solutions, as described below.

The following operations were carried out under conditions designed to insure asepsis and freedom from the extraneous introduction of endotoxin.

The lyophilized residue from 1.2 ml. of lysate was reconstituted by the addition of 1.2 ml. of water, followed by gentle swirling for a few seconds to complete the dissolution of the residue. Aliquots (0.1 ml.) of the reconstituted lysate were dispensed into a series of small test tubes, which were then incubated at 37° C. in a water bath until temperature equilibrium was reached. To each tube was added 0.1 ml. of one of a series of serially diluted endotoxin standards prepared from either *E. coli* endotoxin or FDA Standard *K. pneumoniae* endotoxin. The tubes were swirled gently to mix the solutions and were then incubated undisturbed at 37° C. for 60 minutes. At the end of this time each tube was observed for the occurrence of gelation by gently and slowly tilting the tube a few degrees. If gelation was observed, the tube was rotated through an angle of 180° or until the gel began to distort. A gel was rated "firm" if 180° rotation produced no

distortion. A partial gel which distorted upon rotation of the tube through angles up to 180° was rated "viscous".

The test results for endotoxin concentrations below certain indicated levels are set forth in tables 2 and 3. When tests were run at higher endotoxin levels than those shown in the tables, firm gels were formed in all cases.

Sensitivity of Three Lots of Lyophilized Limulus Amebocyte Lysate After Storage At Elevated Temperatures For Varying Periods of Time

Note: The notation "G" indicates that a firm gel was formed, "V" indicates a viscous gel, and "N" indicates that no gel was formed. "—" indicates that no test was run.

TABLE 2

Storage Temperature 30—32° C.

Lysate Lot No.	Appearance of Dry Residue	Day No.	GELATION RESULTS E. Coli Endotoxin (ng/ml) 0.1	0.05	0.01	FDA Endotoxin (ng/ml) 0.78	0.39	0.19
U	White	0	G	G	N	G	G	N
	,,	1	G	G	N	G	G	G
	,,	2	G	G	N	G	V	—
	,,	3	G	G	N	G	V	—
	,,	4	G	G	N	G	V	V
	,,	5	G	G	N	V	V	—
	,,	6	G	N	N	V	N	—
	,,	7	G	N	N	N	N	—
A	,,	0	G	G	N	G	G	V
	,,	1	G	G	N	G	V	N
	,,	2	G	G	N	G	N	—
	,,	3	G	G	N	G	N	—
	,,	4	G	G	N	G	V	N
	,,	5	G	N	N	V	N	N
	,,	6	G	N	N	N	N	N
	,,	7	N	N	N	N	N	N
L	,,	0	G	G	N	G	G	N
	,,	1	G	G	G	G	G	G
	,,	2	G	G	N	G	G	N
	,,	3	G	G	N	G	G	N
	,,	24	G	G	V	G	G	N
	,,	28	G	G	N	G	G	N

TABLE 3

Storage Temperature 35—37° C.

Lysate Lot No.	Appearance of Dry Residue	Day No.	GELATION RESULTS E. Coli Endotoxin (ng/ml) 0.1	0.05	0.01	FDA Endotoxin (ng/ml) 1.56	0.78	0.39	0.19
U	White	0	G	G	N	G	G	G	N
	,,	1	G	G	V	G	G	G	V
	,,	2	G	G	N	G	G	N	—
	,,	3	G	V	N	G	G	N	—
	,,	4	G	N	N	G	N	N	N
	X*	5	G	V	N	V	V	N	N
	,,	6	N	N	N	N	N	N	N
	,,	7	N	N	N	N	N	N	N
A	White	0	G	G	N	G	G	G	V
	,,	2	G	G	N	G	G	N	—
	,,	3	G	V	N	G	G	N	—
	X*	4	G	V	N	G	G	N	N
	Off-White	5	G	V	N	V	N	N	N
	,,	6	N	N	N	N	N	N	N
	,,	7	N	N	N	N	N	N	N
L	White	0	G	G	N	G	G	G	N
	,,	1	G	G	G	G	G	G	G
	,,	2	G	G	N	G	G	G	N
	,,	3	G	G	N	G	G	G	N
	,,	25	G	G	N	G	G	G	N
	,,	28	G	G	N	G	G	G	N

X* = White to Off-White.

WHAT WE CLAIM IS:—

1. A reagent composition for detecting endotoxin which comprises a lyophilized mixture of Limulus amebocyte lysate and an inert, water-soluble organic stabilizing agent.

2. A composition as claimed in claim 1 wherein the stabilizing agent is a crystallizable sugar or polyhydric alcohol.

3. A composition as claimed in claim 2 wherein the crystallizable sugar is a disaccharide.

4. A composition as claimed in claim 3 wherein the sugar is lactose.

5. A composition as claimed in claim 4 wherein the proportion of lactose is in the range of from 0.01 g to 0.1 g of lactose per 100 ml. of Limulus amebocyte lysate.

6. A composition as claimed in any one of the preceding claims which additionally contains a small proportion of a divalent cation.

7. A composition as claimed in claim 6 wherein the divalent cation is calcium.

8. A composition as claimed in claim 6 or claim 7 wherein the concentration of the divalent cation is in the range of 0.0001 to 0.1 molar.

9. A composition as claimed in any one of the preceding claims which additionally contains a small proportion of a monovalent cation.

10. A composition as claimed in claim 9 wherein the monovalent cation is sodium.

11. A composition as claimed in claim 9 or claim 10 wherein the concentration of the monovalent cation is in the range of 0.001 to 0.1 molar.

12. A composition as claimed in claim 1 substantially as hereinbefore described.

13. A method for preparing a reagent composition for detecting endotoxin which comprises preparing a Limulus amebocyte lysate solution containing an inert, water-soluble organic stabilizing agent and lyophilizing the said solution.

14. A method as claimed in claim 13 wherein the stabilizing agent is a crystallizable sugar or a polyhydric alcohol.

15. A method as claimed in claim 14 wherein the crystallizable sugar is a disaccharide.

16. A method as claimed in claim 15 wherein the sugar is lactose.

17. A method for preparing a reagent composition for detecting endotoxin which comprises preparing a Limulus amebocyte lysate solution containing from 0.01 to 0.1 g of lactose per 100 ml. of Limulus amebocyte lysate and lyophilizing the said solution.

18. A method as claimed in any one of claims 13 to 17 wherein the solution additionally contains a small proportion of a divalent cation.

19. A method as claimed in any one of claims 13 to 18 wherein the solution additionally contains a small proportion of a monovalent cation.

20. A method as claimed in claim 13 substantially as hereinbefore described with reference to the foregoing Example.

21. A reagent composition whenever prepared by a process as claimed in any one of claims 13 to 20.

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